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SUSCEPTIBILITY

Ivermectin Impairs the Development of Sexual and Asexual Stages of *Plasmodium falciparum In Vitro*

Lais Pessanha de Carvalho,[a,b,c] Thaisa Lucas Sandri,[a,b,d] Edésio José Tenório de Melo,[c] Rolf Fendel,[a,b,e] Peter G. Kremsner,[a,b,e] Benjamin Mordmüller,[a,b,e] Jana Held[a,b,e]

[a]Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany
[b]German Centre for Infection Research, Tübingen, Germany
[c]Laboratory of Cell and Tissue Biology, Center for Biosciences and Biotechnology, State University of North Fluminense Darcy Ribeiro, Rio de Janeiro, Brazil
[d]Laboratory of Molecular Immunopathology, Clinical Hospital, Federal University of Paraná, Curitiba, Brazil
[e]Centre de Recherches Médicales de Lambaréné, Lambaréné, Gabon

ABSTRACT Ivermectin is the drug of choice for many parasitic infections, with more than one billion doses being distributed in onchocerciasis programs. The drug has been put into focus recently by the malaria community because of its potential to kill blood-sucking mosquitoes, thereby reducing malaria transmission. However, the activity of ivermectin against the malaria parasite itself has been only partly investigated. This study aimed to investigate the *in vitro* activity of ivermectin against asexual and sexual stages of *Plasmodium falciparum*. Both asexual and late-stage gametocytes were incubated with ivermectin and control drugs *in vitro*. The growth-inhibiting effects were assessed for asexual stages of different *Plasmodium falciparum* laboratory strains and culture-adapted clinical isolates using the histidine-rich protein 2 enzyme-linked immunosorbent assay technique. The effect against stage IV/V gametocytes was evaluated based on ATP quantification. Ivermectin showed activities at nanomolar concentrations against asexual stages (50% inhibitory concentration of ~100 nM) and stage IV/V gametocytes (500 nM) of *P. falciparum*. Stage-specific assays suggested that ivermectin arrests the parasite cycle at the trophozoite stage. Ivermectin might add a feature to its "wonder drug" properties with activity against asexual stages of the malaria parasite *Plasmodium falciparum*. The observed activities might be difficult to reach with current regimens but will be more relevant with future high-dose regimens under investigation. Further studies should be performed to confirm these results *in vitro* and *in vivo*.

KEYWORDS *Plasmodium falciparum*, antimalarial agents, gametocyte

Ivermectin, a mixture of two semisynthetic analogs, is a macrocyclic lactone derived from avermectin B_1, a compound that is naturally produced by the bacterium *Streptomyces avermitilis*; it is considered one of the most successful drugs discovered (1). It acts robustly at low doses, with a broad spectrum of activity against nematodes, insects, and acarine parasites, and has a very good safety profile (2). Ivermectin was discovered in the early 1970s and was licensed in 1981 as an antiparasitic drug for animal health (3); since 1987, it has been used to treat onchocerciasis in affected human populations around the tropics (4). Ivermectin has shown unprecedented versatility, safety, and benefits primarily in the world's poorest areas (5). Currently, ivermectin is used to treat other tropical diseases, such as onchocerciasis, lymphatic filariasis, and strongyloidiasis (2), and it is also active against scabies and lice (6, 7). Recently, ivermectin has been under investigation as a complementary tool for blocking and controlling malaria transmission (8, 9).

Malaria is a mosquito-borne parasitic disease, with approximately 219 million cases

Citation de Carvalho LP, Sandri TL, José Tenório de Melo E, Fendel R, Kremsner PG, Mordmüller B, Held J. 2019. Ivermectin impairs the development of sexual and asexual stages of *Plasmodium falciparum in vitro*. Antimicrob Agents Chemother 63:e00085-19. https://doi.org/10.1128/AAC.00085-19.

Address correspondence to Jana Held, janaheld@hotmail.de.

L.P.D.C. and T.L.S. are co-first authors.

Received 14 January 2019
Returned for modification 13 February 2019
Accepted 9 May 2019
Accepted manuscript posted online 20 May 2019
Published 25 July 2019

TABLE 1 Results of the asexual growth inhibition assay for chloroquine and ivermectin, presented as IC_{50} values

Parasite strain	IC_{50} (mean ± SD) (nM)	
	Chloroquine	Ivermectin
3D7	3.3 ± 1.5	100 ± 32.1
Dd2	206.9 ± 52.7	110 ± 42.2
JH1	103 ± 20.6	21.5 ± 6.9
JH13	111.2 ± 21	126.4 ± 40.6
JH26	10.9 ± 4.2	137.9 ± 38.2
K1	321.7 ± 72.1	365.3 ± 92.3

resulting in 435,000 deaths worldwide in 2017 (10) and with the greatest burden in sub-Saharan Africa, especially in young children (11). Five species of *Plasmodium* can cause human malaria, but *Plasmodium falciparum* infections are responsible for most of the deaths (12). Currently, artemisinin-based combination therapies (ACTs) are the recommended treatment for uncomplicated malaria in all regions in which the disease is endemic (13). ACTs appeared as a solution for drug-resistant *P. falciparum* malaria, with rapid elimination of asexual parasites and young gametocytes (14, 15). However, ACTs are ineffective against mature gametocytes, allowing persistent parasite transmission after treatment (16). Besides using potent antimalarial drugs against the blood-stage parasites, blocking through vector control or utilizing transmission-blocking drugs is essential for the effective control of malaria transmission. Insecticide-treated nets and indoor residual spraying are used extensively, although vectors can develop resistance to insecticides (17) or bite in outdoor areas. In addition, the emergence of *P. falciparum* parasites that are less susceptible to artemisinin derivatives, especially in Southeast Asia (14, 18), calls for the development of new antimalarial drugs and demonstrates the need for additional methods to complement malaria control. Recently, several studies investigated the potential of ivermectin for malaria control, especially focusing on its capacity to inhibit parasite transmission (19–21) and sporogony (22–24) and its insecticidal activity (25–29). However, *in vitro* studies regarding the effects of ivermectin against the sexual and asexual stages of the parasite are still lacking. Thus, the aim of this study was to investigate the *in vitro* activity of ivermectin against asexual and mature gametocyte stages of *P. falciparum*.

RESULTS

Ivermectin inhibits the development of asexual parasites at nanomolar concentrations. Ivermectin was active against all tested laboratory strains and clinical isolates, with a 50% inhibitory concentration (IC_{50}) of ~100 nM (Table 1). The clinical isolate JH1 was approximately 5 times more sensitive and the K1 strain was 3 times more resistant to ivermectin than the other tested strains. This effect was not correlated with chloroquine resistance. Ivermectin had dose-dependent activities against all parasite strains. Drastic parasite growth inhibition occurred with all strains (Fig. 1).

Ivermectin arrests the parasite cycle at the trophozoite stage. Samples were collected every 12 h and subsequently were stained and examined by light microscopy, to investigate the effect of ivermectin on the intracellular parasites. As shown in Fig. 2, the assay started (0 h) when the synchronized parasite culture was at the ring (Fig. 2a.1), trophozoite (Fig. 2c.1), or schizont (Fig. 2e.1) stage. Ivermectin-treated ring-stage parasites arrested development at the trophozoite stage, as can be seen in Fig. 2b.2. Controls, i.e., parasites without drug, developed normally through the cycle (rings, trophozoites, schizonts, and rings again) (Fig. 2a.1 to a.7) until the end of the assay (72 h). In comparison, the parasites treated with 100 nM ivermectin developed to trophozoites but were not able to transform into schizonts, presenting a pyknotic morphology until the end of the assay (Fig. 2b.3 to b.7).

The same procedures were performed with synchronized trophozoites (Fig. 2c.1 to c.7 and Fig. 2d.2 to d.7) and synchronized schizont-stage parasites (Fig. 2e.1 to e.7 and

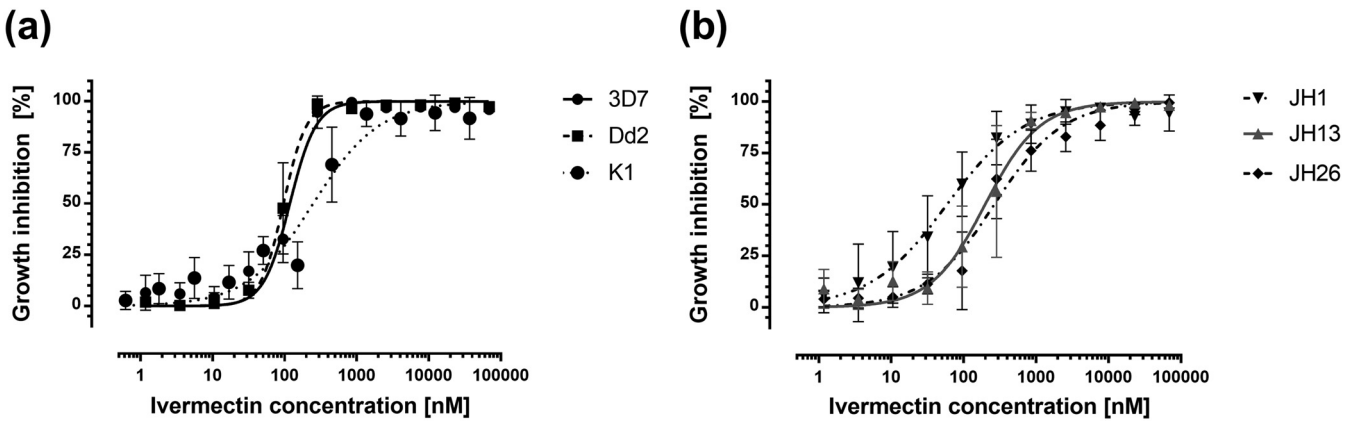

FIG 1 Inhibition curves for different *Plasmodium falciparum* strains in the presence of ivermectin. Mean inhibition curves with SDs for laboratory strains (a) and clinical isolates (b) are shown.

Fig. 2f.2 to f.7). In all assays, parasites arrested at the trophozoite stage irrespective of the stage that was first subjected to the drug (Fig. 2).

Ivermectin is active against mature gametocytes at nanomolar concentrations. Table 2 shows the IC_{50} values of the drugs chlorotonil A (internal control), methylene blue and epoxomicin (positive controls), and ivermectin against mature gametocytes, revealing that ivermectin also had some activity against this stage of the parasite, although a 5-fold higher concentration was needed, compared to the asexual assay.

DISCUSSION

Ivermectin exhibits unprecedented antiparasitic effects, with activity against helminths, insects, and arachnids (30). Since the approval of the use of ivermectin in humans, the drug has been tested against a variety of human parasitic diseases, as reviewed previously (31, 32), but it was suggested that it was inactive against bacteria, fungi, flatworms, and protozoans (33). However, recent publications described *in vitro* activity of ivermectin against flavivirus (34), bacterial species (35–37), *Toxoplasma gondii* (38), and *Trypanosoma brucei* (39).

Recently, ivermectin was discovered as a potential drug for malaria transmission control (8, 19, 40–42) and it has been investigated due to its insecticidal effects (25–27, 43) and its ability to inhibit *Plasmodium* sporogony (24). Additionally, ivermectin was tested against *Plasmodium berghei* liver stages *in vitro* and *in vivo* (44); it inhibited the development and replication of the parasite inside human hepatoma cells (Huh7) *in vitro* with an IC_{50} of ~2 μM (2.1 μg/ml). In addition, ivermectin reduced the *P. berghei* liver infection in mice by 80% after 46 h of treatment with three doses of 10 mg/kg. Overall, it was stated that ivermectin impaired liver-stage development of *P. berghei* and subsequently decreased the blood parasite burden, thereby improving survival time for infected mice.

Despite many studies investigating ivermectin as a promising drug for malaria transmission control, there are few available data regarding the *in vitro* effects of ivermectin on asexual and sexual *P. falciparum* stages. One group tested ivermectin *in vitro* against the *P. falciparum* K1 strain and found only low activity (IC_{50} of 8 μg/ml, corresponding to 9.1 μM) (45). However, the present study showed a higher activity of ivermectin against parasites of the K1 strain, with an IC_{50} value of 365 nM, which is 24 times less than the concentration observed previously. Differences with respect to our results might be partly explained by methodological differences, e.g., differences in measurement of parasite viability (hypoxanthine assay versus histidine-rich protein 2 [HRP2] assay), addition of the drug (dissolved in methanol and dried versus diluted in dimethyl sulfoxide [DMSO] followed by complete culture medium), and parasitemia conditions. No other publications assessing the *in vitro* activity of ivermectin against *P. falciparum* are available. Panchal and collaborators (46) evaluated the mode of action

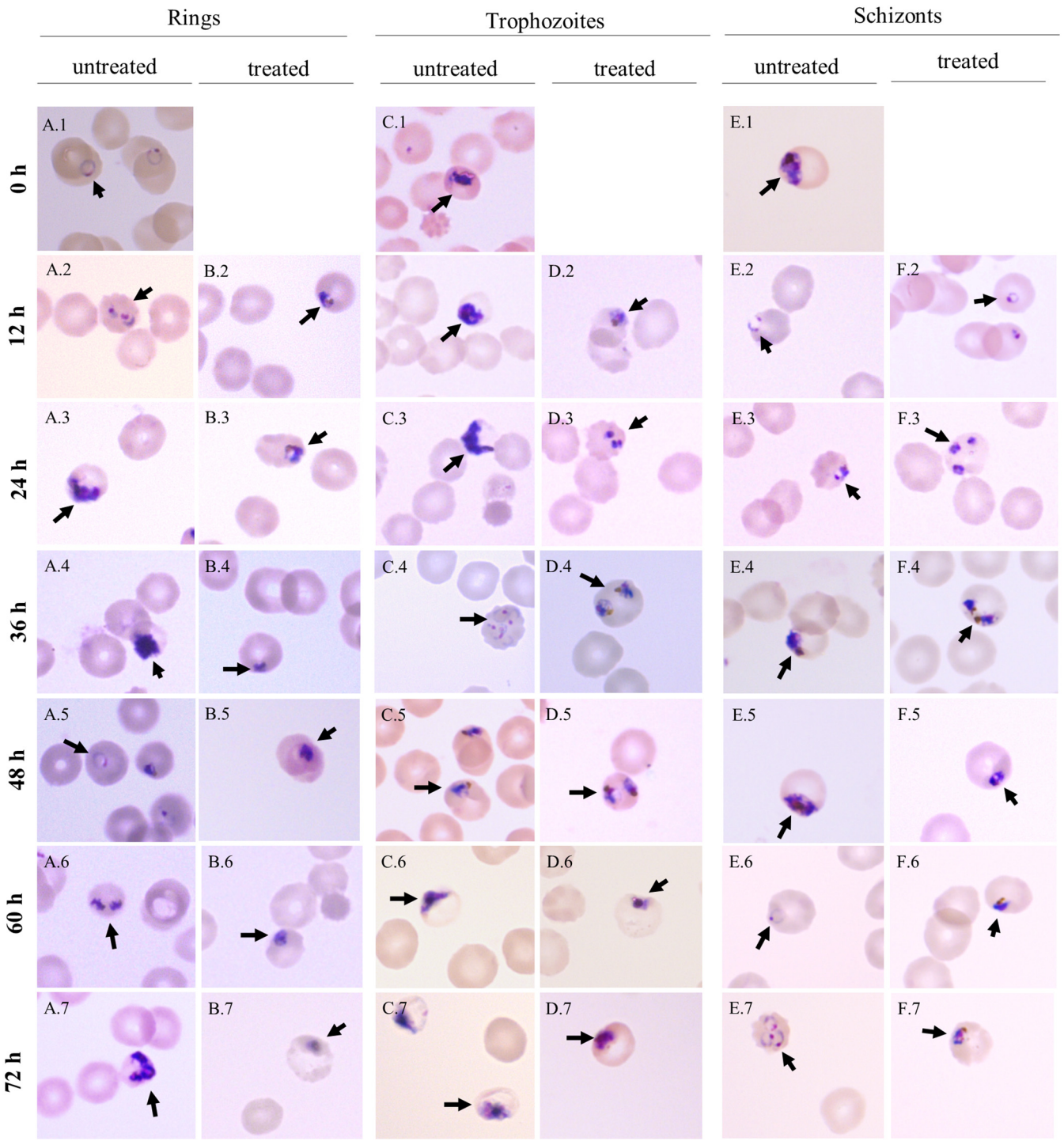

FIG 2 Light microscopy of parasite cultures. Synchronized stages of *Plasmodium falciparum* strain 3D7 were treated with ivermectin for 72 h. Samples were evaluated by microscopy every 12 h until 72 h, to observe the parasites' morphology. Untreated parasites in the ring (a.1 to a.7), trophozoite (c.1 to c.7), or schizont (e.1 to e.7) stage were maintained in parallel. Ivermectin-treated ring-stage parasites became trophozoites, but parasites did not develop further to schizonts (b.2 to b.7). Ivermectin-treated trophozoites arrested their life cycle, not completing maturation to schizonts (d.2 to d.7). Schizonts treated with ivermectin released merozoites to infect new erythrocytes; the merozoites developed to rings and also arrested at the trophozoite stage (f.2 to f.7). All treated parasites were exposed to 100 nM ($\sim IC_{50}$ value). In summary, ivermectin arrested the parasite cycle at the trophozoite stage, and parasites started to present a condensed and pyknotic morphology.

of ivermectin against *P. falciparum* laboratory strains (3D7 and Dd2) when investigating the effects of nuclear import/export inhibitors on *P. falciparum* signal recognition particles (SRPs). They used concentrations ranging from 10 to 25 μM for 24 h but did not determine IC_{50} values. We found *in vitro* activity of ivermectin against the intra-

TABLE 2 IC_{50} values for different drugs against mature gametocytes of *P. falciparum*

Drug	IC_{50} (mean ± SD) (nM)
Chlorotonil A	6.43 ± 3.6
Methylene blue	284.9 ± 203.4
Epoxomicin	2.7 ± 1.7
Ivermectin	558.7 ± 103.9

erythrocytic stages of both laboratory strains and three clinical strains isolated in Gabon (JH1, JH13, and JH26) at nanomolar concentrations (Table 1). In addition, some activity against mature gametocytes (NF-54 strain) was seen (Table 2).

The intraerythrocytic cycle of *P. falciparum* takes approximately 48 h, during which it shows different morphological stages, including the ring stage, the trophozoite stage (the most metabolically active stage), and the multinucleated schizont stage (47). The present study suggests that ivermectin is stage specific, arresting the parasites' life cycle at the trophozoite stage, when they become pyknotic, and inhibiting their development into schizonts. Schizont maturation and merozoite invasion of uninfected erythrocytes seemed microscopically not to be affected. This arresting effect was also seen by Panchal et al. (46), which suggested that this effect on parasite growth could be attributed to the blockage of nucleocytoplasmic shuttling of SRP polypeptides, avoiding the signal sequence recognition needed for protein synthesis. Most of the drugs used against *Plasmodium* sp., such as chloroquine, amodiaquine, mefloquine, and piperaquine, target trophozoites (48–50). Artemisinins additionally target ring stages as well as schizonts, so that all asexual blood stages are affected (49, 50). Hemoglobin-digesting parasite stages also presented a condensed and pyknotic morphology after treatment with chloroquine and artemisinin (47).

Most antimalarial drugs have no activity against mature gametocytes, allowing the transmission of malaria after treatment (51). The administration of drugs such as mepacrine, quinine, and sulfadoxine-pyrimethamine has been associated with the stimulation of gametocytemia, potentially increasing transmission to mosquitoes (52, 53). Other drugs, such as artemisinin and atovaquone, are known to be effective only against early gametocyte stages (54–56). Only a few compounds have shown effects against mature gametocytes *in vivo* in humans, including methylene blue (57), the 8-aminoquinoline primaquine (58), and tafenoquine (16). The mode of action of primaquine in gametocytes is unclear, but it has been suggested that it targets mitochondrial function (59). The observed *in vitro* activity of ivermectin against mature gametocytes probably will not translate into pronounced *in vivo* activity, as the necessary plasma levels will not be reached due to dosing constraints. However, the prominent activity against blood-sucking mosquitoes might be complemented and affect transmission.

Pharmacokinetic studies showed that, after oral administration of the recommended ivermectin dose in humans (200 μg/kg), the concentration in plasma reaches 50 ng/ml (60), a concentration that would not have a major impact on parasite survival, according to our *in vitro* data. In this study, the IC_{50} values against the asexual stages for most of the parasite strains were ~100 nM, corresponding to 87 ng/ml. However, plasma levels of ivermectin can vary greatly, depending on drug administration conditions. When a high-fat meal is given immediately before ivermectin administration, the drug absorption increases (61, 62). A concentration above the IC_{50} could be maintained for 12 h when a single oral dose of 30 mg of ivermectin was given with food (61); therefore, the *in vitro* inhibitory effects could be translated to *in vivo* effects against the disease-causing asexual stages when the appropriate dosing and administration are used. Moreover, ivermectin availability was greater when the drug was administered in a hydroalcoholic solution, in comparison with a solid capsule formulation or a tablet (63). With the oral dose (400 μg/kg) recommended by French authorities to control lymphatic filariasis (64), ivermectin plasma concentrations could reach up to 260 ng/ml when the drug was given with food, with a time to the maximal plasma concentration

of 4.9 ± 1.8 h (61). Guzzo et al. (61) showed that, under fasted conditions, concentrations of 165.2 ± 98.6 ng/ml were reached after administration of one dose of 60 mg ivermectin and concentrations of 186.2 ± 130.8 were detected on day 7 after three doses of 60 mg. Ivermectin has a large volume of distribution (3.1 to 3.5 liters/kg) due to its high lipid solubility and its extensive protein binding (unbound fraction of <0.1), and the peak plasma concentration occurs approximately 4 h after oral dosing, with a second peak at 6 to 12 h, probably arising due to enterohepatic recycling, and a plasma half-life of ~12 h (60, 65, 66). A recent study investigating the malaria-transmission-blocking potential of ivermectin used concentrations of 300 μg/kg or 600 μg/kg given on 3 consecutive days (total dose of 900 μg/kg or 1,800 μg/kg, respectively) in a fasted state, reaching plasma concentrations of around 69.4 ng/ml (range, 34.1 to 196.3 ng/ml) or 118.9 ng/ml (range, 45.2 to 455.1 ng/ml), respectively, and showed that these doses were well tolerated and reduced mosquito survival rates for at least 28 days after treatment (67, 68). Results from the different pharmacokinetic investigations show that concentrations of ivermectin that affect parasite survival can be reached, depending on the chosen administration method and dosing. Different formulations (for example, slow-release ivermectin) are options that have to be further investigated if ivermectin is to be considered an additional transmission-blocking tool (69).

The ability to decrease mosquito survival rates several days after drug intake is the key advantage of ivermectin in malaria control and can have a huge impact on residual transmission. Some vector species changed their behavior to evade the indoor residual spraying of insecticides and the use of long-lasting insecticidal nets. Although these two methods are efficient in controlling malaria transmission while humans are asleep indoors at night, atypical attacks on humans in the mornings or evenings when unprotected people are outdoors allow for residual transmission (70). At this point, ivermectin, although it cannot protect the individual from being bitten, avoids transmission of the parasite (22).

In conclusion, ivermectin showed promising *in vitro* activity against asexual blood stages of two laboratory (3D7 and Dd2) and three clinical (JH1, JH13, and JH26) *P. falciparum* strains, in addition to some activity against mature gametocytes. Further investigations are necessary to confirm these results and to determine whether this activity can also be shown *in vivo*. If this is the case, an additional feature can be appended to the exceptional activity profile of ivermectin, adding to its properties as a complementary tool for malaria control and elimination.

MATERIALS AND METHODS

Chemicals. The positive-control drug for the asexual assay was chloroquine diphosphate (Sigma) and those for the gametocyte assay were epoxomicin (Sigma), methylene blue (Sigma), and chlorotonil A (provided by the Helmholtz Centre for Infection Research and Pharmaceutical Biotechnology, Saarland University, Saarbrücken, Germany) (71). Stock solutions of ivermectin (Sigma product no. I8898; molecular mass of 875.1 g/mol) and epoxomicin were diluted in DMSO at 100 mM and 1 mM, respectively. Methylene blue and chloroquine were dissolved in distilled water at 13 mM and 1 mM, respectively, and chlorotonil A was dissolved in tetrahydrofuran at 2.1 mM. All compounds were first diluted to a stock solution before further dilution in complete medium to reach the desired start concentration. Final solvent concentrations in the assay (<0.1%) did not interfere with parasite growth. Pilot experiments showed that DMSO displays no toxicity when concentrations are <0.5%. The ivermectin used here has the same composition as the ivermectin commonly used for mass drug administration (Mectizan and Stromectol; B1a, ≥90%; B1b, ≤5%).

Asexual stage parasite culture. *P. falciparum* laboratory strains 3D7 (chloroquine sensitive), Dd2 (chloroquine, sulfadoxine, and pyrimethamine resistant), K1 (chloroquine, sulfadoxine, and pyrimethamine resistant), and NF-54 (provided by Sanaria; a vigorous gametocyte producer) and clinical isolates from Gabon JH1 (chloroquine resistant), JH13 (chloroquine resistant), and JH26 (chloroquine sensitive) (72) were maintained in continuous culture as described previously (73). Briefly, parasites were kept in complete culture medium (RPMI 1640 medium [Sigma], 2 mM L-glutamine [Gibco], 12 ml of 1 M HEPES [Gibco], 50 μg/ml gentamicin [Gibco], and 0.5% [wt/vol] AlbuMax II) at 37°C in 5% CO_2/5% O_2 at 2.5% hematocrit, with a change of medium every 2 days and with regular parasite dilution with fresh erythrocytes. The 3D7, Dd2, K1, JH1, JH13, and JH26 strains were used in the growth inhibition assays with the asexual stages, while NF-54 was used to produce mature gametocytes. Regular synchronization was performed using magnetic column separation.

Gametocyte culture. Gametocyte culture was performed as described previously (74), with minor modifications. *P. falciparum* NF-54 strain synchronized ring-stage parasites were used to initiate the

gametocyte culture at 6% hematocrit and 0.3% parasitemia. Complete culture medium (as described above) supplemented with 5% human serum was changed daily, without parasite dilution, for 14 days. The medium was doubled when parasitemia reached 5%, and 50 mM *N*-acetyl-D-glucosamine (MP Biomedicals GmbH) was added between day 11 and day 14, to remove asexual stages. On day 15, when most of the gametocytes had reached maturity (stage IV/V), the gametocytes were purified with a NycoPrep 1.077 cushion density gradient and magnetic column separation, to remove erythrocytes and to concentrate the gametocyte population (75).

***In vitro* growth inhibition assay for ring-stage asexual *P. falciparum* parasites.** The drug assays were performed as described by Noedl et al. (76). Briefly, ivermectin and chloroquine were distributed in a 3-fold serial dilution in 96-well plates before synchronized ring-stage parasites were diluted to a parasitemia of 0.05% with blood group O Rh factor-positive erythrocytes and were seeded at a hematocrit of 1.5% in a total volume of 225 μl per well. After 72 h, the plates were frozen and thawed three times, and an enzyme-linked immunosorbent assay for measurement of HRP2 was performed using a microplate reader (PHOmo; Autobio). All experiments were performed in duplicate and repeated at least three times.

***In vitro* activity of compounds against late-stage (stage IV/V) gametocytes of *P. falciparum*.** Drug assays were performed as described previously (75). The compounds (ivermectin, epoxomicin, methylene blue, and chlorotonil A) were precoated in a 3-fold dilution in 96-well plates. Subsequently, the previously purified stage IV/V gametocytes were added to the plate (50,000 gametocytes/well) and incubated at 37°C in 5% CO_2/5% O_2. After 48 h, the ATP production of gametocytes was measured by the BacTiterGlo assay (Promega), according to the manufacturer's instructions, and results were quantified using a luminometer (LUmo; Autobio). All experiments were repeated at least three times in duplicate.

Stage-specific activity of ivermectin. To investigate whether the effect of ivermectin was stage specific, the asexual parasites were synchronized to the ring stage using magnetic column separation. The rings were used readily or after the development to trophozoites (18 h) or schizonts (30 h). The stage-specific parasites were seeded in 96-well plates as described above, with modification of the parasitemia (1%) to facilitate observation by light microscopy. Every 12 h of drug incubation, one sample was collected from the control well (no drug) and the 100 nM ivermectin-treated well (IC_{50} value for 72 h of treatment). Thin blood smears were prepared, fixed in pure methanol for 10 s, and stained in Giemsa (Merck) solution (5%) for 20 min. The samples were visualized using Leica DMBL microscopy, and pictures were taken using a ProgRes C10 camera and software (Jenoptik, Germany), at ×100 magnification.

Statistics. Individual IC_{50} values were determined by nonlinear regression analysis of log concentration-response curves, using the drc v0.9.0 package (77) of R v2.3.1 (78). Mean IC_{50} values and standard deviations (SDs) were calculated for each drug assayed in laboratory strains and clinical isolates. Graphical presentation of the drug inhibition data was performed using GraphPad Prism v7.04 for Windows (GraphPad Software, La Jolla, CA, USA).

ACKNOWLEDGMENTS

We acknowledge support by the Conselho Nacional de Pesquisa, with a scholarship to L.P.D.C. Institutional funding supported this work.

We declare no conflicts of interest.

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